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Potential metabolism of pharmaceuticals in radish: Comparison of *in vivo* and *in vitro* exposure

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ABSTRACT

Metabolism of pharmaceuticals in plants is important to evaluate their fate and accumulation in vegetables, and subsequently the risks to human health. However, limited knowledge is available to evaluate the metabolism of pharmaceuticals in plants due to the lack of appropriate research approaches. In this study, radish was selected as a model plant to investigate the metabolism of pharmaceuticals in intact plants (*in vivo*) growing in hydroponic solution and in plant tissue enzyme extracts (*in vitro*). For caffeine, six phase-I demethylation metabolites identified in the intact radish plant were also found in the plant enzyme extracts. After 7 days of *in vivo* exposure, the amount of the identified metabolites was about 5.4 times greater than the parent caffeine in radish roots. Furthermore, the metabolism potential of fifteen pharmaceuticals in radish was evaluated on the basis of mass balance. After 7 days of hydroponic exposure, oxytetracycline, trimethoprim, carbamazepine, lincomycin, monensin and tylosin manifested relatively less extent of metabolism with the mass recoveries ranging from 52.3 to 78.2%. In contrast, 17 β-estradiol, sulfamethoxazole, sulfadiazine, estrone, triclosan, acetaminophen, caffeine, carbadox and lamotrigine underwent extensive metabolism with only 3.0 to 32.1% of the parent compound recovered. In the *in vitro* system, 17 β-estradiol, estrone, triclosan, oxytetracycline, acetaminophen, sulfadiazine and sulfamethoxazole were readily metabolized in radish root

enzyme extracts with 1.8 to 34.0% remaining after 96-h exposure. While in the leaf enzyme extracts, only triclosan was rapidly metabolized with 49.2% remaining, and others pharmaceuticals were ≥ 60%, indicating that the varying extents of metabolism occurred in different plant parts. This study highlights the importance of pharmaceutical metabolism in plants, and suggests that plant tissue enzyme extracts could serve as an alternative tool to assess pharmaceutical metabolism in plants.

Capsule: Similar metabolism patterns were observed for rapidly metabolized pharmaceuticals in both *in vivo* (radish tissue enzyme extracts) and *in vivo* (the intact plant) exposure.

Keywords: Plant metabolism; Plant uptake; Pharmaceuticals; Plant tissue enzyme extracts.

1. Introduction

Reclaimed water reuse in agricultural irrigation has been increasingly practiced to alleviate the burden of water scarcity, especially in arid and semiarid regions (Bischel et al., 2011; Sato et al., 2013). Biosolids and animal manures are also commonly applied to agricultural lands for their fertility values, and as a convenient disposal approach as well (Clarke and Smith, 2011; Cogger et al., 2013; Kumar et al., 2005). These practices also release pharmaceuticals to the environment, resulting in their ubiquitous presence in soils with concentrations at ng kg^{-1} to μg kg^{-1} levels (Chen et al., 2014; Durán-Alvarez et al., 2009; Kinney et al., 2006; Vazquez-Roig et al., 2010). These pharmaceuticals could enter vegetables from soils via root uptake, which serves as the starting point in the food chain of human and animal dietary consumption. It has been well documented that vegetables could accumulate a range of pharmaceuticals from soils (Malchi et

al., 2014; Prosser and Sibley, 2015; Tanoue et al., 2012; Wu et al., 2013). However, only a few attempts have been made to elucidate the metabolism of pharmaceuticals in plants. In addition to uptake and translocation, metabolic transformation of pharmaceuticals is also an important process influencing their residue levels in plants and their potential risk of food safety.

Pharmaceuticals could be metabolized in plants by multiple pathways and form a variety of transformation products (Macherius et al., 2012; Miller et al., 2016; Riemenschneider et al., 2017). In general, the metabolic processes involve three phases of reactions. Pharmaceuticals could be mediated by plant enzymes and form more polar and water-soluble products via phase I reactions (oxidation, reduction and hydrolysis). In phase II reactions, pharmaceuticals and their phase-I metabolites could be conjugated to endogenous plant biomolecules such as amino acids, sugars and glutathione, and form relatively larger-sized molecules. Compared to their parent compounds these conjugates generally demonstrate an enhanced water solubility and higher mobility in plants. These conjugates formed by phase II reactions could be sequestered in plant vacuoles or cell walls by phase III reactions (He et al., 2017; Macherius et al., 2012; Sandermann, 1992). The metabolic transformations of some pharmaceuticals in plants have been studied such as triclosan (Macherius et al., 2012), benzotriazole (LeFevre et al., 2015), ibuprofen (He et al., 2017), diclofenac (Huber et al., 2012), iopromide (Cui et al., 2017) and carbamazepine (Riemenschneider et al., 2017). Carbamazepine was moderately metabolized in tomato after 35 days of exposure, 21 phase I and II transformation products were identified, and their total amount was equivalent to ~ 45% of carbamazepine accumulated in plants (Riemenschneider et al., 2017). Among these metabolites, the 10,11-epoxycarbamazepine manifested even greater toxic potency than carbamazepine (Tomson et al., 1990). In many cases, the amount of formed metabolites exceeded that of the parent compounds remaining in plants. For instance, the sum of

eight phase II triclosan conjugates was 5 times greater than triclosan in carrots during two-month growth in greenhouse (Macherius et al., 2012). In another study, three major metabolites of benzotriazole were found to be >1.5 times more than benzotriazole after 8-day exposure in hydroponic *Arabidopsis* (LeFevre et al., 2015). Besides the intact plants, the metabolism of pharmaceuticals was also studied using plant cell cultures (Fu et al., 2017; Huber et al., 2009; Macherius et al., 2012; Marsik et al., 2017; Sauvêtre et al., 2018; Wu et al., 2016). Triclosan, naproxen, diclofenac, ibuprofen, gemfibrozil, sulfamethoxazole and atorvastatin were found to rapidly disappeared in carrot cell cultures with 0.4 to 47.3% remaining after 90 h of exposure (Wu et al., 2016). The lack of assessment of metabolism could underestimate the total accumulation of pharmaceuticals and metabolites in plants and the potential risk related to the consumption of contaminated agricultural products. Paltiel et al. (2016) recently reported that human consumption of fresh produce irrigated with reclaimed water could lead to the appearance of bioactive metabolites of carbamazepine in their urine.

In vivo exposure (intact plants) is an effective way to examine the fate and metabolism of pharmaceuticals in plant-water (or -soil) systems. However, the intact plant experiments conducted in hydroponic solution or soil are usually labor-intensive and time-consuming. The presence of microorganisms and root exudates also influence pharmaceutical metabolism, which cannot be excluded from the *in vivo* studies (Yu et al., 2013; Zhang et al., 2016). The uptake and transport processes could further complicate the metabolism of pharmaceuticals in plants by governing the input and output of parent compounds/metabolites in a specific plant part. In plants, enzyme-mediated transformation has been considered as a major process to the metabolism of xenobiotics in plants (Huang et al., 2013; Van Eerd et al., 2003). Plant enzyme extracts have been used in the *in vitro* system to investigate the enzyme-mediated metabolism of

polybrominated diphenyl ethers (Huang et al., 2013), estrogens (Card et al., 2013) and metformin (Cui and Schröder, 2016). These studies showed that the metabolites from the reactions with plant enzyme extracts were similar to the products formed in the intact plants. Therefore, plant enzyme extracts may serve as an alternative system to provide a simple and quick approach to evaluate the metabolism of pharmaceuticals in plants, which is minimally affected by plant microbial, uptake and transport processes.

The objective of this study was to compare the pharmaceutical metabolism in radish using both *in vitro* and *in vivo* experiments. Radish was selected because this root vegetable is usually consumed in raw, and might represent the worst case of exposure scenarios. Caffeine was selected as one representative pharmaceutical for further evaluation of the formed metabolites in both intact radish and radish tissue enzyme extracts because it is one of the most frequently detected pharmaceuticals in vegetables (Goldstein et al., 2014; Malchi et al., 2014; Wu et al., 2014; Wu et al., 2013). In addition, we expanded the experiments to fifteen commonly-used pharmaceuticals with a wide range of physicochemical properties to assess their metabolism potential in radish. This study provides new knowledge on the metabolic transformation of pharmaceuticals in plants. The *in vitro* exposure method could serve as an alternative to quickly screen the metabolism potential of pharmaceuticals in plants.

2. Materials and methods

2.1. Chemicals and regents

Fifteen pharmaceuticals were purchased from Sigma-Aldrich (St. Louis, MO, USA) including acetaminophen, caffeine, carbamazepine, sulfadiazine, sulfamethoxazole, lamotrigine,

117 carbadox, estrone, 17 β-estradiol, triclosan, trimethoprim, lincomycin, oxytetracycline, monensin
118 and tylosin. These pharmaceuticals were selected because they are frequently detected in the
119 environment, and demonstrate a wide range of physicochemical properties (Table S1). Caffeine
120 metabolites xanthine, 3-methylxanthine, 7-methylxanthine, theophylline, paraxanthine and
121 theobromine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ceramic homogenizers,
122 octadecylsilane (C18), primary secondary amine (PSA), and graphitized carbon black (GCB)
123 were purchased from Agilent Technologies (Santa Clara, CA, USA). Disodium
124 ethylenediaminetetraacetate (Na_2EDTA), formic acid, glacial acetic acid, and sodium chloride
125 (NaCl) were purchased from J.T. Baker (Phillipsburg, NJ, USA). All organic solvents were of
126 HPLC grade from Fisher (Fair Lawn, NJ, USA). Water used in this study was produced from a
127 Milli-Q water purification system (Billerica, MA, USA).
128
129 *2.2. In vivo hydroponic experiment*
130 Radish seeds (*Raphanus sativus,* Burpee & Co., Warminster, PA) were germinated on
131 moistened filter paper for 4 days. The seedlings were then transferred to a plastic container with
132 10 L of nutrient solution (pH 6.5) (Hydrodynamics International, Lansing, MI, USA). After 14
133 days of growth, the radish plants (~10 cm in height) were used in the exposure experiments.
134 Each radish plant was carefully transferred to a 250-mL glass jar containing 230 mL of nutrient
135 solution with spiked pharmaceuticals. This exposure experiment was conducted with two
136 treatments (1) the radish was exposed to 200 ng mL^{-1} of caffeine in nutrient solution; (2) the
137 radish was exposed to a mixture of fifteen pharmaceuticals with the initial concentration of 100
138 ng mL^{-1} for each compound. The solution was continuously aerated via Teflon tubing connected
139 to a fusion air pump. The jars were wrapped with aluminum foil to prevent the potential

140 photolysis of pharmaceuticals and to minimize algae growth. Pharmaceutical-free controls
141 (radish only) and pharmaceutical solution controls (without radish) were also conducted in the
142 experiments. All controls and exposure experiments were performed in triplicate. During the
143 experiment, nutrient solution (without pharmaceuticals) was added daily to each jar to
144 compensate the water loss from plant transpiration. The glass jars were placed in a controlled
145 growth chamber at 20 ± 2 °C. The light cycle was set up as 10 h of fluorescent light every day
146 with the intensity of 150 mmol/m^2/s followed by 14 h of darkness. For the treatment with only
147 caffeine, radish roots, leaves and solution were sampled at day 1, 3 and 7. For the exposure of the
148 mixture of pharmaceuticals, aqueous solution, radish roots and leaves were collected after 7 days
149 of growth. The radish samples were thoroughly rinsed with deionized water, wiped with tissue
150 paper, weighed, chopped into small pieces, freeze-dried and ground to fine powders. All samples
151 were stored in freezer at −20 °C prior to the extraction of pharmaceuticals.
152
153 *2.3. In vitro reactions with radish enzyme extracts*
154 Pharmaceutical metabolism was also examined *in vitro* using the crude enzyme extracts
155 from radish tissues. Radish enzyme extracts were prepared using the method reported by Card *et*
156 *al.* (Card et al., 2013). In brief, fresh radish tissues were separated into roots and leaves, chopped
157 into small pieces, quickly frozen, ground into powders in liquid nitrogen, and then extracted with
158 50 mM potassium phosphate buffer (pH 7.0) at the mass to solution ratio of 1: 2 (g: mL) in ice
159 for 30 min. The homogenate was centrifuged at 8000 g for 40 min at 4 °C. The supernatant of
160 tissue enzyme extracts was collected, passed through a 0.22-μm cellulose ester membrane
161 (Millipore, Cork, Ireland), and immediately stored in an ice bath prior to use.

The *in vitro* exposure experiment was performed by mixing 200 ng mL^{-1} of caffeine or a mixture of fifteen pharmaceuticals at 100 ng mL^{-1} for each chemical with the radish enzyme extracts. The reactant mixtures were incubated in 1.5-mL centrifuge tubes in a water bath (25 °C), and the reaction was quenched by adding 50 μL of glacial acetic acid. The experiments were conducted in triplicate, along with enzyme extracts-free controls. The resultant solution was sampled at the time intervals of 0, 4, 10, 24, 48, 72 and 96 h. An aliquot of the solution (0.5 mL) was diluted to 1.0 mL with methanol and subject to pharmaceutical analysis. The proteins concentration in radish enzyme extracts was used to estimate the activity of the enzyme mixture, which was measured during the experiment using Bradford assay (Bradford, 1976). The proteins concentration in the radish enzyme extracts demonstrated relatively stable during 96 h of reaction (Figure S1).

2.4. Analysis of pharmaceuticals

Pharmaceuticals in radish root and leaf samples were extracted using a modified QuEChERS method (Chuang et al., 2015). The detailed extraction procedure is given in the supporting information (SI). Hydroponic solution was filtered through a 0.22-μm cellulose ester membrane, and an aliquot of the filtrate (0.5 mL) was diluted to 1.0 mL with methanol and injected directly into a LC-MS/MS system. The LC-MS/MS system consisted of a Shimadzu prominence high-performance liquid chromatography (Columbia, MD, USA) coupled to a Sciex 4500 triple quadrupole mass spectrometer (Foster City, CA, USA), and a 50 mm × 2.1 mm Agilent C18 column (Torrance, CA, USA). The optimized conditions and quality assurance of the LC-MS/MS is detailed in SI. Multiple reaction monitoring (MRM) parameters are listed in

Tables S2 and S3. The corresponding matrix recoveries and method detection limits (MDLs) for the studied pharmaceuticals are summarized in Tables S4-S7.

The metabolism of pharmaceuticals in radish enzyme extracts was fit to the first-order kinetic model $C_i = C_0 e^{-kt}$, where C_i and C_0 is pharmaceutical concentration at sampling time t (h) and the beginning time, respectively, and k is the rate constant (h^{-1}). The dissipation half-life ($T_{1/2}$) was calculated as $T_{1/2} = \ln 2/k$.

3. Results and discussion

3.1. Biotransformation of caffeine in hydroponic system

The mass distribution of caffeine in radish leaves, roots and hydroponic solution is shown in Figure S2. On the basis of the initial amount of caffeine added to the solution, 11.8, 48.9 and 82.6% disappeared in the system after 1, 3 and 7 days of exposure, respectively. The quick depletion of caffeine in the solution, compared to the unplanted controls (< 5% of loss after 7 days), suggests that caffeine could be taken up by radish and undergoes relatively rapid transformation in the hydroponic system. Six demethylation metabolites from phase I reactions were identified and quantified using their authentic standards including xanthine, 3-methylxanthine, 7-methylxanthine, theophylline, paraxanthine and theobromine. All six metabolites were detected in both radish roots and leaves. None of the metabolites was found in the radish-free controls indicating that the metabolism is plant-associated. Only a small amount of xanthine was found in the hydroponic solution with radishes at day 3 and 7, and no other metabolites were found in the solution (Table S8). The presence of xanthine in the solution could be attributed to the excretion from plants and/or caffeine transformation by root exudates.

206 The molar fractions of the six metabolites increased with exposure time in both roots and
207 leaves (Figure 1). In the radish roots, the parent compound caffeine gradually decreased from
208 67.1% at day 1 to 49.8% at day 3, and to 15.6% at day 7, indicating that caffeine was
209 substantially metabolized. The product xanthine (loss of three $–CH_3$ groups from caffeine) was
210 the most abundant metabolite with the molar fraction increasing from 16.7% at day 1 to 58.5% at
211 day 7. Theobromine (loss of one $–CH_3$ group) was the major intermediate with the fraction
212 increasing from 9.0% at day 1 to 13.2% at day 7. This time-dependent transformation pattern
213 was also observed for 3- and 7-methylxanthine with their molar fractions up to 5.3% at the end
214 of experiment. Theophylline and paraxanthine (loss of one $–CH_3$ group) were the least abundant
215 metabolites with the molar fractions < 3.8% (day 3); their fractions increased between day 1 and
216 3 and then decreased from day 3 to 7. Compared with the metabolism occurring in radish roots,
217 caffeine experienced less transformation in the leaves (Figure 1). The parent compound caffeine
218 was predominant with the molar fraction of 94.6% at day 1, 77.5% at day 3, and 68.1% at day 7.
219 Xanthine was the most abundant metabolite at day 1 (2.2%) and day 3 (9.6%), and theobromine
220 was most abundant at day 7 (9.9%). Given that these phase I reaction products all contained the
221 moiety of xanthine that maintains the bioactivity of the parent compound (Goth and Cleaver,
222 1976), the extensive biotransformation of caffeine in radish, particularly in the edible root
223 fraction, could lead to an underestimate of the risk associated with food safety if only
224 considering the presence of the parent compound.
225
226 *3.2. Transformation of caffeine in radish enzyme extracts*
227 The results of the *in vivo* hydroponic experiment provided the information on the
228 metabolism of caffeine in the intact radish. However, the uptake and translocation of caffeine in

229 radish, as well as radish growth, could add more confounding impacts to elucidating the
230 metabolism process. The *in vitro* exposure experiments with radish enzyme extracts offer an
231 alternative to investigate the metabolism of pharmaceuticals in plants. The amount of caffeine
232 and its metabolites formed in the radish tissue enzyme extracts as a function of time is shown in
233 Figure 2. During 96-h exposure, the loss of caffeine in root enzyme extracts was 29.9%, which
234 was approximately 2 times that in leaf enzyme extracts (15.4%) (Figure 2). Meanwhile, the total
235 amount of metabolites formed by demethylation reactions in root enzyme extracts was
236 approximately 2 times that in leaf enzyme extracts, suggesting that a larger extent of
237 biotransformation occurred in radish roots. This is consistent with the results of the *in vivo*
238 hydroponic experiment in which the larger fraction of metabolites was formed in radish roots
239 than in leaves. The amount of the six demethylation metabolites continued to increase over time
240 in both root and leaf enzyme extracts, except for xanthine in the leaf enzyme extracts which
241 decreased slightly from 72 h to 96 h. The amount of metabolites formed in both tissue enzyme
242 extracts at 96 h of exposure ranked in the order of xanthine > theobromine > 3-methylxanthine
243 $\approx$ 7-methylxanthine > theophylline $\approx$ paraxanthine (Figure 2), which is also consistent with the
244 ranking of demethylation metabolites found in the *in vivo* intact radish. In the *in vivo* hydroponic
245 experiment, caffeine accumulation in roots and leaves varied with time due to the continuous
246 water movement into radish; therefore, it is not feasible to compare the absolute amount of the
247 formed metabolites between the *in vitro* and *in vivo* experiments. However, the consistence in the
248 types of metabolites and their rankings in magnitude in both radish roots and leaves suggests that
249 the *in vitro* exposure to plant tissue enzyme extracts could provide a convenient alternative to
250 evaluate the metabolism of pharmaceuticals in a specific plant compartment (Card et al., 2013;
251 Cui and Schröder, 2016).

252 In both *in vivo* and *in vitro* studies, phase I demethylation reaction is identified as a
253 metabolism pathway for caffeine in radish (Figure 3). The possible transformation route follows
254 stepwise N-demethylation reactions via the loss of one $–CH_3$ group to form paraxanthine,
255 theobromine and theophylline, loss of the second $–CH_3$ group to form 7- and 3-methylxanthine,
256 and loss of all three $–CH_3$ groups to form the end product xanthine. The initial N-demethylation
257 reaction (loss of one $–CH_3$ group) is common in mammalians and insects, which is believed to be
258 mediated by cytochrome P450 enzyme systems (i.e. CYP1A2 isoenzyme) (Berthou et al., 1991;
259 Berthou et al., 1992; Coelho et al., 2015; Kot and Daniel, 2008). The N-demethylation reaction
260 of antidiabetic agent metformin was also observed in *Typha latifolia* (Cui and Schröder, 2016).
261 P450 enzymes are also commonly present in plants, and catalyze many metabolic reactions of
262 xenobiotics including some pharmaceuticals and herbicides (Fonne-Pfister et al., 1988; Huber et
263 al., 2009; Siminszky, 2006; Thies et al., 1996). Caffeine is believed to undergo similar enzyme-
264 mediated transformation in radish. In mammalians paraxanthine represents the large fraction of
265 the primary metabolites (70-80%) during the first step of demethylation (Berthou et al., 1991;
266 Kalow and Tang, 1993; Kot and Daniel, 2008). However, in this study theobromine was the most
267 abundant intermediate in radish.
268
269 *3.3. Mass distribution of pharmaceuticals in hydroponic system*
270 Pharmaceuticals have been frequently detected in vegetables grown in fields amended
271 with biosolids or irrigated with reclaimed water (Christou et al., 2017; Malchi et al., 2014; Wu et
272 al., 2014); however, their metabolism in plants still remains largely unknown. To obtain more
273 basic information on the metabolism of pharmaceuticals in vegetables, we further assessed
274 metabolism potential of fifteen pharmaceuticals in radish on the basis of mass balance using the

hydroponic experiment. The mass recoveries of pharmaceuticals in the radish-free controls ranged from 92.0 to 108.2% indicating that pharmaceuticals in nutrient solution were relatively stable, with the only exception of oxytetracycline (14.5%) (Table S9). The substantial loss of oxytetracycline could be due to the rapid hydrolysis in the nutrient solution at pH 6.5 (near neutral), which is the most favorable condition for the hydrolysis of oxytetracycline (Xuan et al., 2009).

The mass distribution of the fifteen pharmaceuticals in solution, roots and leaves after 7-d exposure is presented in Figure 4. The mass recoveries varied between 3.0 and 78.2% of the initial pharmaceutical input, with the relatively high recoveries for tylosin (78.2%), monensin (76.4%), lincomycin (74.3%), carbamazepine (68.4%), trimethoprim (58.6%) and oxytetracycline (52.3%). Among these six pharmaceuticals, the majority of trimethoprim (36.7%), lincomycin (69.4%), monensin (54.5%), and tylosin (43.8%) remained in the nutrient solution, whereas large fractions of oxytetracycline (27.1%) and carbamazepine (40.8%) were accumulated in radish plants. The metabolism of carbamazepine in vegetables such as sweet potato, carrot, tomato, and cucumber could produce 10,11-epoxycarbamazepine and 10,11-dihydroxycarbamazepine (Goldstein et al., 2014; Malchi et al., 2014). Riemenschneider *et al.* (2017) found 21 phase-I and II transformation products derived from carbamazepine in tomato after 35 days of exposure, and accounted for ~45% of carbamazepine uptake into plant. The recoveries were relatively low for other nine pharmaceuticals ranging from 3.0 to 32.1%, only 3.0-4.0% for 17 β-estradiol, sulfamethoxazole, sulfadiazine and estrone, 11.7-18.4% for triclosan, acetaminophen and caffeine, 20.7 and 32.1% for carbadox and lamotrigine. Similar metabolism potential of triclosan was also observed in carrot by Macherius et al. (2012), who identified 8 triclosan conjugation products and their total amounts was about 5 times more than the parent

298 compound triclosan. The total recovered mass was < 80% for all tested pharmaceuticals after 7
299 days of exposure, and the metabolism in radish is expected to be responsible for such
300 discrepancies between the initial input and the remaining amount. The potential risk originating
301 from the metabolites are still unclear, particularly for those quickly and intensively metabolized
302 pharmaceuticals such as estrogens and sulfonamide antibiotics.
303
304 *3.4. Metabolism of pharmaceuticals in radish enzyme extracts*
305 The metabolism potential of the fifteen pharmaceuticals in radish root and leaf enzyme
306 extracts was examined by measuring their dissipation during 96 h of exposure (Figure 5). In root
307 enzyme extracts the tested pharmaceuticals demonstrated a wide range of variation in mass
308 recoveries from 1.8 to 98.1% of the initial dosage (Figure 5). Lincomycin, monensin, tylosin,
309 carbadox, carbamazepine, lamotrigine, trimethoprim and caffeine showed relatively low to
310 minimal transformation with the mass recoveries ranging between 72.3 and 98.1%.
311 Carbamazepine and trimethoprim demonstrated high stability in an *in vitro* study, where their
312 concentration remained nearly unchanged in carrot cell cultures during the 90 h of exposure, and
313 < 5% of carbamazepine was transformed after 22 days of incubation (Wu et al., 2016). In another
314 lately *in vitro* study, about 5% of carbamazepine was transformed in horseradish hairy root cell
315 cultures after 6 days of exposure (Sauvêtre et al., 2018). Sulfamethoxazole, sulfadiazine,
316 acetaminophen, oxytetracycline, triclosan, estrone and 17 β-estradiol manifested a rapid
317 metabolism with the recovered amount of < 34.0% of the initially spiked dosage. The fast
318 metabolism of estrogens, acetaminophen, sulfamethoxazole and triclosan is consistent with
319 previous *in vitro* studies (Card et al., 2013; Huber et al., 2009; Macherius et al., 2012; Wu et al.,
320 2016). Three conjugates (acetaminophen–glucoside, acetaminophen-glutathione, and cysteine

conjugate) of acetaminophen in the cell cultures of *A. rusticana* were identified after 6 h of exposure, and their total amounts was about 4.6 times that of acetaminophen (Huber et al., 2009). Wu et al. (2016) found that about 55.5 and 91.7% of triclosan and sulfamethoxazole disappeared in carrot cell cultures within 90 h. Macherius et al. (2012) reported that ~95% of triclosan was quickly metabolized within 24 h in carrot cell cultures. In this study, the rapidly depleted pharmaceuticals were found for sulfamethoxazole, sulfadiazine, acetaminophen, oxytetracycline, triclosan, estrone and 17 β-estradiol in both intact radish and root enzyme extracts. However, the highly metabolized carbadox and caffeine in intact radish showed less depletion in the enzyme extracts.

The extent of metabolism of pharmaceuticals in leaf enzyme extracts was less than that in root extracts with the exception of carbadox and lamotrigine (Figure 5). Among the pharmaceuticals studied, triclosan demonstrated the highest transformation in leaf enzyme extracts (50.8%); however, the remaining fraction was still ~5 times greater than that in root enzyme extracts (9.5%). Sulfadiazine, carbadox and sulfamethoxazole were moderately metabolized in leaf enzyme extracts with the remaining fractions of 59.4%, 61.4% and 72.1%, respectively. For the remaining ten pharmaceuticals, the majority of the initially spiked pharmaceuticals still existed in the leaf enzyme extracts (79.2-98.8%) (Figure 5). The different metabolism rates of pharmaceuticals between root vs leaf enzyme extracts could be related to the types and amount of extracted enzymes, and their metabolic activities in different radish tissues. The changes in metabolism magnitude could alter the distribution and accumulation of pharmaceuticals in different plant parts, especially in the scenario that pharmaceuticals enter plants by root uptake. For instance, only 1.8% of 17 β-estradiol remained in root enzyme extracts after 96-h exposure, while > 90% of 17 β-estradiol was still present in leaf enzyme extracts

344 (Figure 5). In the *in vivo* experiment, 17 β-estradiol concentration in the leaves was 16 ng g^{-1}
345 which was >10 times greater than that in roots (1.4 ng g^{-1}). The results from the *in vitro*
346 experiments clearly support the rapid metabolism of 17 β-estradiol in radish roots, which is
347 believed to lead to the corresponding lower concentration in roots in the *in vivo* experiments.
348 Pharmaceuticals in different plant parts could have varying metabolism rates such as
349 carbamazepine in sweet potato, carrot, and tomato (Malchi et al., 2014; Riemenschneider et al.,
350 2017) and iopromide in cattail (Cui et al., 2017).

351 The rapid metabolism of sulfamethoxazole, sulfadiazine, acetaminophen, oxytetracycline,
352 triclosan, estrone and 17 β-estradiol in radish root enzyme extracts was fit to the first-order
353 kinetic model, and the fittings are shown as curves in Figure 6 with R^2 = 0.94-0.97 (Table S10).
354 The corresponding half-life was estimated as 17 β-estradiol (15.4 h) < estrone (17.3 h) <
355 triclosan (30.1 h) < acetaminophen (33.0 h) < oxytetracycline (36.5 h) < sulfadiazine (49.5 h) <
356 sulfamethoxazole (63.0 h). The rapid dissipation of triclosan and sulfamethoxazole were also
357 found in the carrot cell cultures with half-life of ~0.2 h and ~7.7 h, respectively (Wu et al., 2016).
358 Compared with the results of carrot cell cultures, triclosan and sulfamethoxazole both exhibited a
359 much slower dissipation rate in the radish enzyme extracts. Triclosan demonstrated a faster
360 dissipation rate than sulfamethoxazole in both *in vitro* systems. The high removal of these
361 pharmaceuticals in root enzyme extracts is consistent with their low mass recoveries in the *in*
362 *vivo* study (Figure 4), suggesting that these biotransformation reactions in root enzyme extracts
363 could also take place in radish roots in the *in vivo* experiments. Application of plant tissue
364 enzyme extracts to react with pharmaceuticals could serve as an appropriate approach for rapid
365 evaluation of their metabolism potential in plants. This particularly simplifies the evaluation of

366 plant compartment-dependent metabolism, and could also eliminate the impact of uptake and
367 translocation to metabolism process in the intact plant experiments.
368
369 **4. Conclusion**
370 The deficient knowledge on metabolism of pharmaceuticals in plants limits the accurate
371 risk assessment of pharmaceuticals accumulated in vegetables because of the lack of appropriate
372 experimental or technique protocols for identification and quantification of metabolites. In this
373 study, the comparison in caffeine metabolism in radish between *in vivo* and *in vitro* exposure
374 experiments demonstrates that the demethylation metabolites identified in radish tissue enzyme
375 extracts (*in vitro* study) were similar to those formed in the intact radish roots and leaves in the *in*
376 *vivo* experiment. Similar metabolism patterns (dissipation of parent compounds) were observed
377 for rapidly metabolized pharmaceuticals including 17 β-estradiol, estrone, acetaminophen,
378 triclosan, oxytetracycline and sulfonamides in both radish tissue enzyme extracts and in the
379 intact plant. These results suggest that the metabolic reactions with plant tissue enzyme extracts
380 could be used as an appropriate approach for rapid examination of metabolism potential of
381 pharmaceuticals in a specific plant tissue, with the minimal impact from uptake and translocation.
382 For those extensively metabolized pharmaceuticals such as 17 β-estradiol, acetaminophen,
383 triclosan and sulfonamides, the measurement of the parent compounds in plants is not sufficient
384 to assess their accumulation and potential risks since the formed metabolites might still carry the
385 bioactive moieties and pose similar adverse impacts to plant and human health. Further
386 investigation of the metabolism of these pharmaceuticals in plants and their related
387 bioactivity/toxicity are needed to warrant the safe use of reclaimed water for irrigation and land
388 application of biosolids and animal manures.

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Appendix A. Supporting Information

Additional details are provide in the Supporting Information including physicochemical properties of pharmaceuticals, analytical methods, mass recovery of pharmaceuticals in the hydroponic controls, protein concentration in enzyme extracts, mass distribution of caffeine in hydroponic-radish system, dissipation curves of pharmaceuticals in radish enzyme extracts, and representative chromatograms of pharmaceuticals.

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